Genome-scale analysis of the cotton *KCS* gene family revealed a binary mode of action for gibberellin A regulated fiber growth

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Abstract Production of β-ketoacyl-CoA, which is catalyzed by 3-ketoacyl-CoA synthase (*KCS*), is the first step in very long chain fatty acid (VLCFA) biosynthesis. Here we identified 58 *KCS* genes from *Gossypium hirsutum*, 31 from *G. arboreum* and 33 from *G. raimondii* by searching the assembled cotton genomes. The gene family was divided into the plant-specific FAE1-type and the more general ELO-type. *KCS* transcripts were widely expressed and 32 of them showed distinct subgenome-specific expressions in one or more cotton tissues/organs studied. Six *GhKCS* genes rescued the lethality of *elo2Δelo3Δ* yeast double mutant, indicating that this gene family possesses diversified functions. Most *KCS* genes with GA-responsive elements (GAREs) in the promoters were significantly upregulated by gibberellin A_3 (GA). Exogenous GA_3 not only promoted fiber length, but also increased the thickness of cell walls significantly. GAREs present also in the promoters of several cellulose synthase (*CesA*) genes required for cell wall biosynthesis and they were all induced significantly by GA_3. Because GA treatment resulted in longer cotton fibers with thicker cell walls and higher dry weight per unit cell length, we suggest that it may regulate fiber elongation upstream of the VLCFA-ethylene pathway and also in the downstream steps towards cell wall synthesis.

INTRODUCTION

Cotton fibers are single-celled trichomes differentiated from the ovule epidermis and considered as a model system for studying cell elongation and cell wall biogenesis (Kim and Triplett 2001; Wang et al. 2004; Pang et al. 2010; Qin and Zhu 2011; Zhu and Li 2013; Li et al. 2014). Many genes are involved in various steps during fatty acid biosynthesis and elongation is highly upregulated during cotton fiber initiation and elongation (Ji et al. 2003; Qin et al. 2005; Shi et al. 2006; Yang Samuel et al. 2006; Gou et al. 2007; Qin et al. 2007; Shang et al. 2013; Zou et al. 2013; Liu et al. 2015). Very long chain fatty acids (VLCFAs) (C20:0 to C30:0) significantly promoted cotton fiber cell elongation with several *KCS* genes highly upregulated during cotton fiber development (Qin et al. 2007).

Very long chain fatty acids are generated in the endoplasmic reticulum with four successive reactions catalyzed by four diverse enzymes: Condensation of a long chain acyl-CoA with a malonyl-CoA by 3-ketoacyl-CoA synthase (KCS); reduction of 3-ketoacyl-CoA by 3-ketoacyl-CoA reductase (KCR); dehydration of 3-hydroxyacyl-CoA by 3-hydroxyacyl-CoA dehydratase (HCD); and further reduction of trans-2-enoyl-CoA by trans-2-enoyl-CoA reductase (ECR) to form a two carbon elongated acyl-CoA (Fehling et al. 1991; Riezman 2007). In yeast, Elo1p is responsible for the elongation of medium-chain fatty acids, Elo2p participates in the elongation to C24, and Elo3p is essential for the elongation of C24 to C26

(Toke et al. 1996; Oh et al. 1997; Dittrich et al. 1998). Yeast cells with *elo2* and *elo3* double deletion is constitutive lethal (Oh et al. 1997). The functions of *KCS* genes in other organisms are often characterized with lethality restoration of the double mutant yeast cell line.

KCS is regarded as the rate-limiting enzyme that also determines the substrate and tissue specificities of fatty acid elongation in higher plants (Lassner et al. 1996; Millar et al. 1997; Denic and Weissman 2007). VLCFAs were found in sphingolipids, seed oil and epicuticular waxes in plant. In the *Arabidopsis* genome, 21 FAE1-type and 2 ELO-type *KCS* genes were reported, with the functions of ELO-type KCSs largely unknown (Dunn et al. 2004; Costaglioli et al. 2005; Joubès et al. 2008). The biological functions and substrate specificities (based on differences in carbon chain length and degrees of unsaturation) of several *KCS* genes have gradually been elucidated in the past several decades using various *Arabidopsis* mutants. For example, FAE1 isolated from the *Arabidopsis thaliana* mutant *fae1* was shown to catalyze C20 and C22 VLCFA biosynthesis for storage lipids in the seeds (Kunst et al. 1992; James et al. 1995; Rossak et al. 2001). CER6/CUT1 was involved in the biosynthesis of C26 and longer VLCFAs for cuticular waxes in epidermis and during root hair development (Hooker et al. 2002; Pang et al. 2010; Xu et al. 2012). Loss of *FDH* (*FIDDLEHEAD1*) function resulted in ectopic organ fusions indicating a crucial role of *KCS* in organ development (Lolle et al. 1997; Yephremov et al. 1999;

Pruitt et al. 2000; Voisin et al. 2009). In addition, complete loss of *KCS20* or *KCS2* function led to the accumulation of C20 VLCFA suggesting that these two *KCSs* may be required for cuticular wax and root suberin biosynthesis (Franke et al. 2009; Lee et al. 2009).

Phytohormones, mainly ethylene, brassinosteroid (BR) and auxin are known to play important roles in cotton fiber cell initiation and elongation (Shi et al. 2006; Zhang et al. 2011). GA was recently reported to be able to regulate cellulose synthesis through the DELLA-NAC signaling cascade in rice (Huang et al. 2015). Exogenous application of GA_3 or indole-3-acetic acid (IAA) to the flowers resulted in significant increases of fiber number per ovule (Seagull et al. 2004) and GA_3 promoted significant fiber cell growth as well (Mei et al. 2010). Previously, GA was suggested to act downstream of the ethylene pathway since the GA biosynthesis inhibitor, paclobutrazol, nullified the biological functions of externally applied ethylene and C24:0, the later is known to mediate ethylene production (Qin et al. 2007; Mei et al. 2010). GAs may function through interaction with GhHOX3, an HD-ZIP IV transcription factor, to upregulate the expression of *GhRDL1* and *GhEXP1* to promote fiber elongation (Shan et al. 2014).

To fully understand the possible functions and the evolution of this important gene family among the three cotton species, we identified 58 *KCS* genes from *G. hirsutum*, 31 from *G. arboreum* and 33 from *G. raimondii*, based on the whole genome sequences (Paterson et al. 2012; Wang et al. 2012; Li et al. 2014, 2015). We focused on the phylogeny and expression pattern analysis, and used the yeast double mutant cell line to characterize systematically the cotton *KCS* genes. We searched for the presence of GAREs and L1 box, which is required for *GhHOX3* function in the GA signaling pathway (Shan et al. 2014), in the promoter regions of *KCS* and *CesA* genes to clarify whether and how GAs are involved in the control of cotton fiber cell growth.

RESULTS

Genome-wide identification of *KCS* genes in three different cotton species

To identify all the *KCS* genes in *G. hirsutum*, *G. arboreum* and *G. raimondii* genome, HMMER and BLAST search were performed using 23 *KCS* genes from *Arabidopsis* and three from yeast as the query. A sum of 47, 27, 29 FAE1-type and 11, 4, 4 ELO-type *KCS* genes were found in *G. hirsutum*, *G. arboreum* and *G. raimondii*, respectively. Among all 58 *KCS* genes identified in the *G. hirsutum* genome, 25 showed At origin and 33 Dt origin (Table S1). Most *G. hirsutum KCS* genes have no intron throughout their whole open reading frames, with only seven possessing one intron and two with two introns.

Phylogenetic analysis of *KCS* genes

Two phylogenetic trees were independently constructed using full length amino acid sequences and the MEGA 5.0 software that employs the neighbor-joining method. All KCS proteins collected from the three cotton species plus the *Arabidopsis* genome fell in two distinct groups, namely the FAE1-type and ELO-type. FAE1-type KCS family proteins were further divided into eight subclasses: α, β, γ, δ, ε, ζ, η and θ (Figure 1A) and the ELO-type KCS proteins were classified

into four different subgroups: I, II, III and IV (Figure 1B). Subgroup η from the FAE1-type and subgroups II, III, IV from ELO-type were only found in *Gossypium*, indicating that *KCS* genes in cotton might have undergone specific gene duplication and have more complicated functions than in *Arabidopsis*. Evolutionary analysis using 19 species from lower aquatic to higher terrestrial plants showed that FAE1-type *KCS* first appeared in *Volvox carteri* and the number dramatically increased in *Physcomitrella patens*, then stayed relatively stable until the allotetraploid cotton evolved (Figure 1C). The ELO-type *KCS* was composed of only one to four genes in all other species analyzed and its number increased dramatically in *G. hirsutum* as well (Figure 1D). This finding is consistent with a whole-genome duplication (WGD) event in the *Gossypium* genus after its separation from *T. cacao* (Wang et al. 2012; Li et al. 2014).

Subgenome-specific transcription activation of 32 *GhKCS* genes

We further investigated the expression patterns of *KCS* genes between the allotetraploid and the corresponding diploid cottons using real-time quantitative polymerase chain reaction (qRT-PCR). Of the 58 analyzed *KCS* genes, 32 genes showed subgenome-specific expression patterns (Figure 2). Furthermore, 26 *KCSs* among the subgenome-specific genes were highly expressed in reproductive organs such as flowers and ovules (Figure 2). Compared to their orthologs from the DD genome, 11 *KCS* genes from Dt subgenome expressed notably higher during various stages of fiber development. Three Dt-derived *KCS* genes showed flower-specific expression, while *DtKCS4* and *DtKCS8* were dramatically expressed in roots. *DtKCS5* was expressed in a stem-specific manner when compared with its ortholog from the DD genome. By contrast, *DDKCS9* was expressed in a highly leaf-preferential manner (Figure 2A). Eight of the At-derived *KCS* genes were markedly higher in different stages of fiber development compared with their orthologs in the AA genome. Four *KCS* genes that originated from the At subgenome were specifically expressed in flowers and two other *KCS* genes were significantly expressed in roots. *AtKCS58* was highly expressed in leaves (Figure 2B). Transcripts of another 15 *KCS* genes either from the At or Dt subgenome showed no subgenome-specificity when compared to their corresponding diploid orthologs and 10 *KCS* genes were always expressed at extremely low levels in the tissues studied (Figure S1). Subgenome-specific high-level expression of various *KCS* genes may somehow be related to the successful development of long and spinnable fibers in the allotetraploid cotton.

Potential regulatory mechanisms of cotton *KCS* genes

Sequence analysis showed that deletions of a 121-bp fragment on *KCS55* and a 237-bp fragment on *KCS43* promoter in the AA genome resulted in the loss of a few MYB binding sites (Figure 3A). Likewise, deletions of two large DNA fragments on *KCS6* promoter from the DD genome resulted in the acquisition of two additional MYC-binding sites on *DtKCS6* promoter (Figure 3A). These changes correlate with the low level expression both in *G. arboreum* and *G. raimondii*, when compared with what was found either in the At or the Dt subgenomes. Also, two additional MYC binding sites on *AtKCS38* promoter and two new MYB binding sites on *DtKCS1*
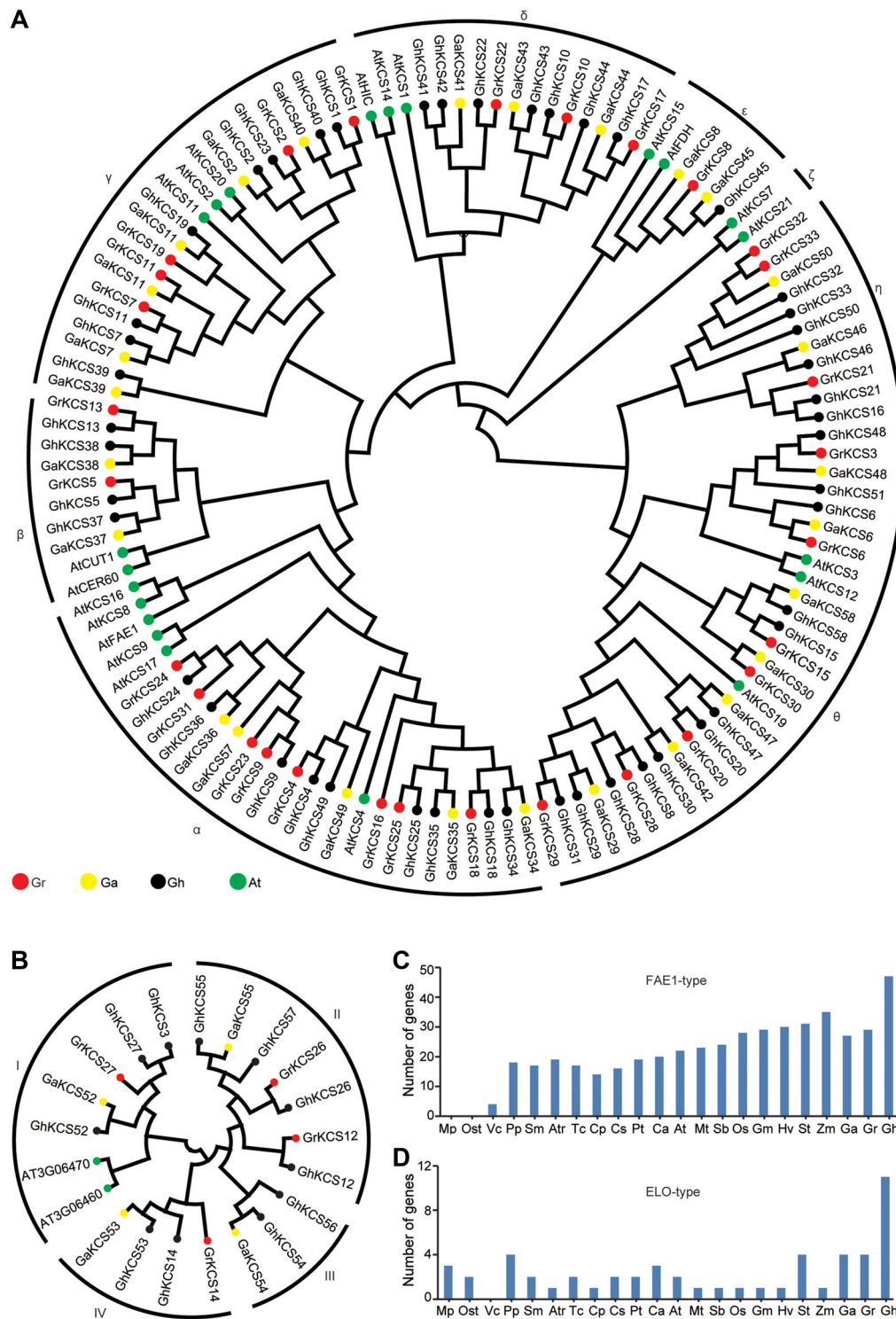
Figure 1. Evolution and diversification of the *KCS* gene family among different organisms
(**A**) The unrooted phylogenetic tree of FAE1-type KCS at the amino acid sequence level using the neighbor-joining method in MEGA 5.0. (**B**) Phylogenetic tree of ELO-type KCS proteins. The color keys are used universally between (**A**) and (**B**). (**C**) Comparisons of FAE1-type *KCS* gene numbers across a wide range of organisms. (**D**) Comparisons of ELO-type *KCS* gene numbers across a wide range of organisms. Mp, *Micromonas pusilla*; Ost, *Ostreococcus tauri*; Vc, *Volvox carteri*; Pp, *Physcomitrella patens*; Sm, *Selaginella moellemdorffii*; Atr, *Amborella trichopoda*; Tc, *Theobroma cacao*; Cp, *Carica papaya*; Cs, *Cucumis sativus*; Pt, *Populus trichocarpa*; Ca, *Capsicum annuum*; At, *Arabidopsis thaliana*; Mt, *Medicago truncatula*; Sb, *Sorghum bicolor*; Os, *Oryza sativa Japonica*; Gm, *Glycine max*; Hv, *Hordeum vulgare*; St, *Solanum tuberosum*; Zm, *Zea mays*; Ga, *Gossypium arboretum*; Gr, *Gossypium raimondii*; Gh, *Gossypium hirsutum*.
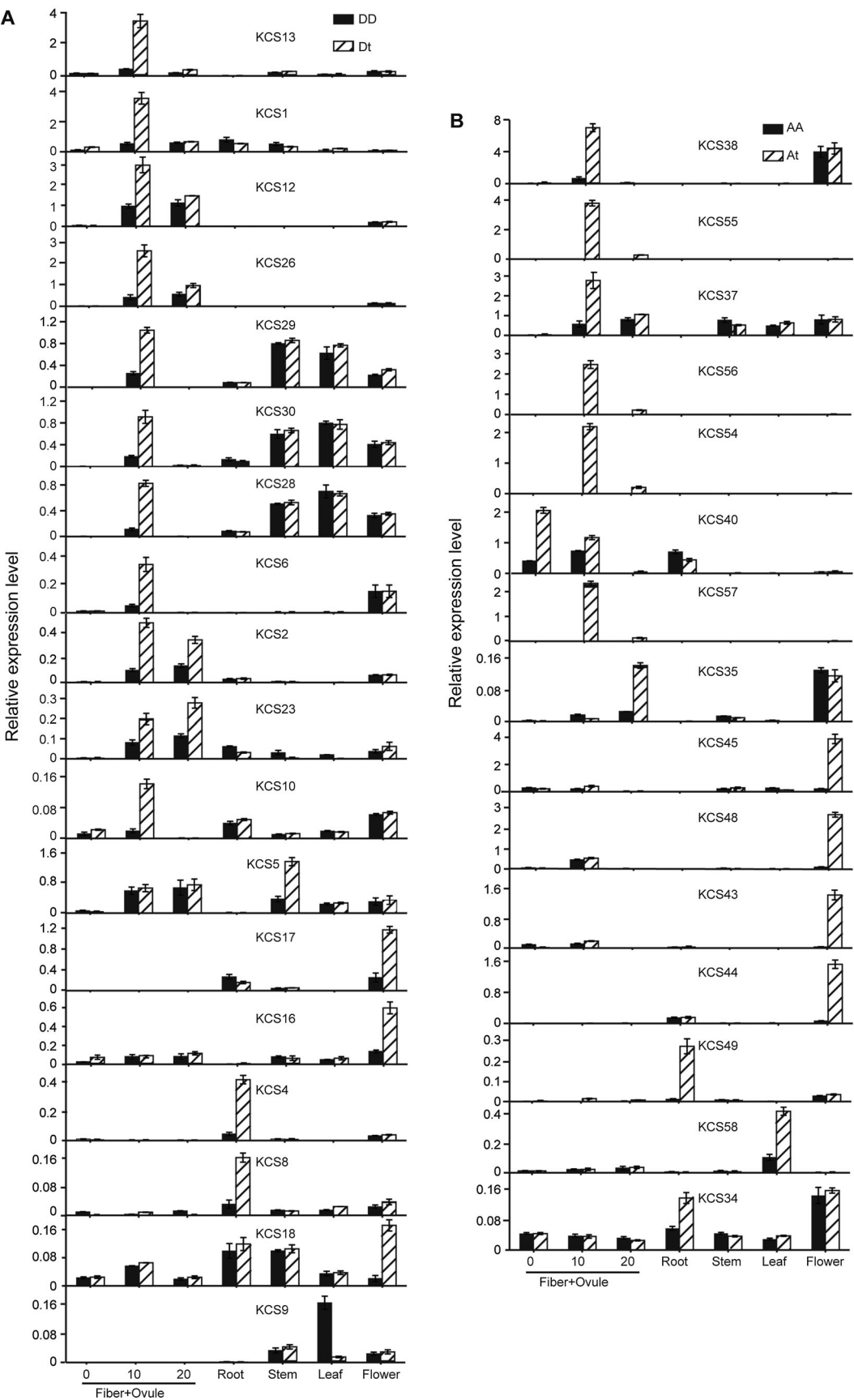
Figure 2. Continued.

promoter were generated potentially as results of point mutations (Figure 3B). The presence of additional or new transcription factor binding sites can be responsible for the highly fiber-specific expression of the Dt or At originated *KCS* genes. Also, we observed four additional GT-1 binding sites on *AtKCS48* promoter (Figure 3B), we suggest that they may facilitate the flower-specific expression from the At-subgenome gene. One each of the *Copia* element, which is a long terminal repeat (LTR) retrotransposon, was inserted in the promoters of *AtKCS44* or *DtKCS28* (Figure 3C). Further, depletion of two WRKY binding sites known to have inhibitory effects on target gene expression, on the *DtKCS18* promoter region, may lead to its high-level flower-specific expression (Figure 3D).

Functional characterization of cotton *KCS* genes

We next constructed *elo2Δelo3Δ* double mutant strain carrying the pYES2-*ScELO3* plasmid. We confirmed the double mutation by successful amplification of *elo2::kanMX4* and *elo3::natMX4* cassettes using genomic DNA prepared from the mutant (Figure 4A). Wild-type and all mutant strains were able to grow on "2% galactose + 0.05% glucose" plates (Figure 4B, top left panel). The *elo2Δelo3Δ* double mutant was unable to grow on 2% glucose medium, owing to the suppression of *ELO3* expression which was driven by galactose (Figure 4B, top right). Both the *elo2* single mutant and the *elo2Δelo3Δ* double mutant were able to grow on geneticin (G418) due to the presence of the *elo2::kanMX4* cassette (Figure 4B, mid left). On the same line, both *elo3* and *elo2Δelo3Δ* were able to grow on Clonat due to the presence of the *elo3::natMX4* cassette (Figure 4B, mid right). Only the *elo2Δelo3Δ* double mutant was able to grow on G418+Clonat plates (Figure 4B, bottom left). By contrast, the double mutant failed to grow on 5-fluoroorotic acid (FOA) medium as a result of the ejection of the pYES2 plasmid, while all other strains grew well (Figure 4B, bottom right). Subsequently, two FAE1-type and four ELO-type KCSs from *G. hirsutum* were found to restore the viability of the double mutant (Figure 5A). *KCS12*, *KCS14*, *KCS53* and *KCS26* produced almost wild-type growth rate as observed on YPD solid plates (Figure 5B, left) and as observed by continuous culture in liquid medium as well (Figure 5B, right). *KCS12* and *KCS26* genes showed fiber-specific expression and *KCS18* was highly expressed in flower (Figure 2), indicating that these *KCS* genes potentially regulated cotton development.

Cells expressing ELO-type cotton *KCS12*, *KCS14*, *KCS53* and *KCS26* genes contained almost identical amounts of C26:0 as compared with the wild-type (Figure 5C; Table S2), indicating that this subgroup may be directly involved in VLCFA biosynthesis up to C26:0, which is the longest fatty acid in yeast cells. Heterologous expression of *KCS18* produced large

amounts of C22:0, with undetectable levels of C24:0 and C26:0. Cells rescued by *KCS20* accumulated high levels of C24:0, with a negligible amount of C26:0 (Figure 5C; Table S2). Considering their low complementation efficiency reported in Figure 5B and their VLCFA accumulation patterns reported in Table S2, we suggest that *KCS18* and *KCS20* are involved in synthesis of C22:0 from C20:0 and C24:0 from C22:0, respectively.

GA3 rapidly promotes the expression of many *KCS* and *CesA* genes during fiber development

When analyzing the promoter regions of all 19 *KCS* genes preferentially expressed during different fiber growth stages, 11 of them were found to contain GAREs with two of them showing L1 boxes as well (Figure 6A). qRT-PCR analysis showed that the transcript levels of eight cotton *KCS*s increased significantly shortly after application of 1 μM GA3 (Figure 6B). For *KCS28* and *KCS23* that have three consecutive GAREs on each promoter, approximately 30-fold increases in transcript levels 24 h after GA3 application was recorded. For *KCS13* and *KCS38* that possessed one GARE and one L1 box on their promoters, about 20–30 fold increases in transcript levels after GA3 treatment were observed (Figure 6A, B). GA3 also induced significant expression of *KCS36* (with two GAREs), *KCS37*, *KCS40* and *KCS1*, which showed one GARE on each of the promoters at greater than 1 kb from the putative translation start sites. Exogenous GA3 failed to activate the transcription of *KCS2*, *KCS35* and *KCS30*, all possessed with one GARE on each of the promoters at less than 1 kb from the putative translation start sites (Figure 6A, B). Further analysis showed that five of the cotton *KCS*s contained BR-insensitive-EMS-suppressor 1 (BES1) elements (Figure S2A) and two contained auxin responsive elements (AuxREs) (Figure S2C). Exogenous applications of BR and auxin induced the transcription of all but one of the *KCS* genes with corresponding elements (Figure S2B, D). GAREs and L1-boxes presented also on *CesA* gene promoters (Figure 6C). qRT-PCR experiments indicated that all four of these *CesA* genes were significantly upregulated by GA3 (Figure 6D). Applications of GA3 did not induce the expression of the other two major *CesA* genes without GARE or L1-box (Figure 6E). Exogenous C26:0 resulted in significant transcript increases for two *CesA* genes (*CesA6-4* and *CesA6-7*), whereas exogenous ethylene did not stimulate the expression of all *CesA* genes (Table S3).

Deciphering the mode of GA actions

When C26:0, ethylene and GA3 were applied to standard ovule culture media for 7 d, significant fiber elongation was observed (Figure 7A). Surprisingly, only exogenous GA3 resulted in significant fiber dry weight increase (calculated

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Figure 2. Genome- or subgenome-specific expression of 33 *KCS* genes

(**A**) Seventeen *KCS* genes from the Dt subgenomes were expressed higher in various tissues when compared to their corresponding diploid orthologs. Black columns indicate the expression of gene from the D genome. Slash columns indicate the expression of gene from the Dt subgenome. (**B**) Fifteen *KCS* genes from the At subgenome were expressed higher in various tissues when compared to their corresponding diploid orthologs. Black columns indicate the expression of gene from the A genome. Slash columns indicate the expression of gene from the At subgenome. qRT-PCR analyses were performed in triplicate using different cotton materials and error bars in this figure represent the SD from three independent experiments.
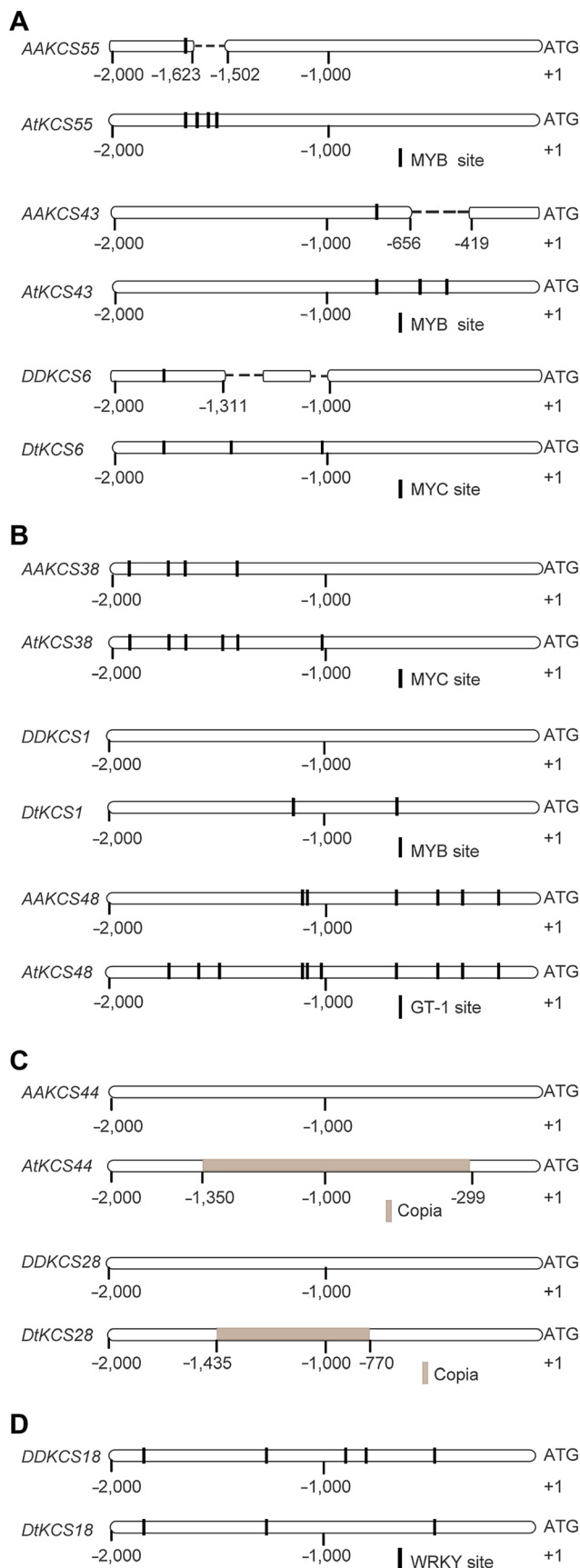
at the per cm bases), whereas C26:0 treatment showed a slight increase of the dry weight and ethylene treated ovules produced similar to untreated wild-type levels (Figure 7B). Fiber cell wall thickness and fiber diameters were measured under an electron microscope using semi-thin sections prepared from fiber cells closest to the ovule epidermal layer. Again, fiber cells harvested from GA$_3$ treated ovules showed most significant cell wall growth, as indicated by both cell wall thickness and by the diameter of each fiber, followed by C26:0 that stimulated cell wall growth to some degree (significant at the 0.05% level) (Figure 7C). Fibers from ethylene treated ovules showed no significant increase in cell wall thickness (Figure 7C). These changes were consistent with significant increase of cellulose content in fibers from GA$_3$ treated ovules, a moderate increase after C26:0 treatment and no increase after ethylene treatment (Figure 7D).

DISCUSSION

The genome of *Arabidopsis* contains 21 FAE1-type *KCS* genes and two ELO-type genes (Costaglioli et al. 2005). In yeast cells, only three ELO-type *KCS* genes have been found so far (Toke and Martin 1996). Here 47 FAE1-type *KCS* and 11 ELO-type *KCS* in *G. hirsutum*, 27 FAE1-type *KCS* and four ELO-type *KCS* in *G. arboreum*, and 29 FAE1-type *KCS* and four ELO-type *KCS* in *G. raimondii* were identified using comparative genomics approaches (Table S1). Since four new subclasses (η, II, III and IV) and many more *KCS* gene family members exist in various cotton species, we suggest that additional KCS functions may be required for this genus and gene duplication events occurred in this plant after its separation from *T. cacao*.

Thirty-two of the 58 *GhKCS* genes displayed subgenome-specific transcription activation over a wide range of tissues studied, with only one *KCS* gene expressed at higher level from *G. raimondii* (the DD genome) in leaves (Figure 2). Interestingly enough, 19 of these *GhKCS* genes were activated at the transcription level during various fiber developmental stages, which indicates that the allotetraploidization event

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Figure 3. Potential regulatory mechanisms of *KCS* genes
(**A**) Deletion of large DNA fragments on various KCS promoter regions resulted in additional transcriptional factor binding sites. Top two drawings: A deletion in *AAKCS55*, but not in its *At* orthologue, produced three additional MYB binding sites in the *AtKCS55* promoter region. Mid two drawings: A deletion in *AAKCS43*, but not in its *At* orthologue, produced two additional MYB binding sites in the *AtKCS43* promoter region. Bottom two drawings: Two deletions in *DDKCS6*, but not in its *Dt* orthologue, produced two additional MYC binding sites in the *DtKCS6* promoter region. (**B**) Point mutations on *KCS* promoter regions from the At or Dt subgenomes, but not their AA or DD orthologs, produced additional or new transcriptional factor binding sites. (**C**) *Copia* elements were found only in *AtKCS44* and *DtKCS28* promoters, but not in their AA or DD orthologs. (**D**) Depletion of two WRKY binding sites on *DtKCS18* promoter region from the Dt subgenome. The same arrangements as outlined in A for all other panels were used in this figure.
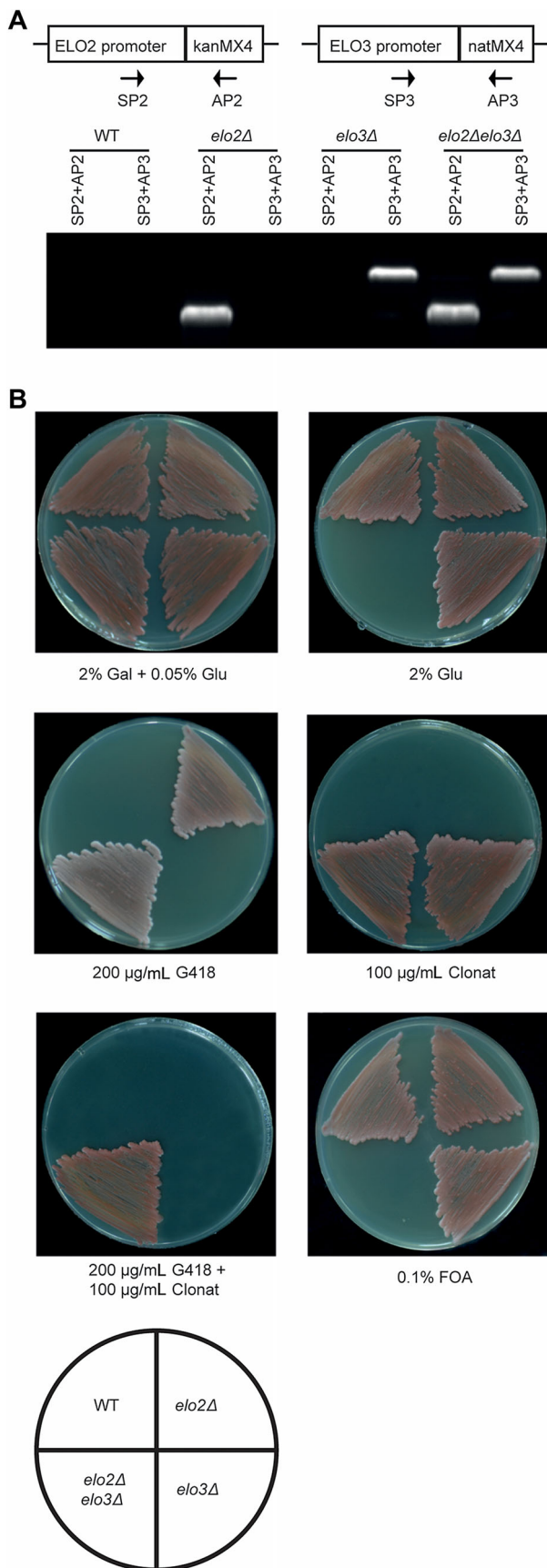
Figure 3. Continued.

plays an important role in regulating *KCS* gene expression and cotton fiber elongation as well. Evolution of more cis-elements and insertion of retrotransposons in nine *KCS* promoter regions of At or Dt subgenomes seemed to have contributed to their highly specific expressions (Figure 3). These results may reflect the necessity of *KCS* genes for proper fiber development in the allotetraploid species. Also, the presence of extra regulatory elements in promoter regions of functionally important genes may be considered resultant of different selection pressures among different cotton species, which was consistent with the previous notion that the allotetraploid genome enjoyed lower selection pressure than the diploid genomes (Li et al. 2015).

Four of the *Arabidopsis* FAE1-type KCS proteins were able to rescue the lethality of *elo2Δelo3Δ* yeast double mutant (Trenkamp 2004; Paul et al. 2006). Complete loss of *KCS20* and *KCS2* decreased the total wax content (Lee et al. 2009) and *CER6/CUT1* mutation affected lipid content on the surface of pollen and stamens in this plant (Fiebig et al. 2000). Currently we find that two FAE1-type and four ELO-type KCSs from *G. hirsutum* were able to substitute the yeast Elo2p/Elo3p proteins for biosynthesis of VLCFA. Mutant yeast cells expressing four of the ELO-type genes including *KCS12*, *KCS14*, *KCS53* and *KCS26* resulted in close-to-wild-type growth rate, whereas markedly lower efficiency was found for cells expressing *KCS18* or *KCS20*, both FAE1-types (Figure 5B). Accordingly, wild-type levels of C26:0 were observed from mutant cells carrying all four ELO-type genes, with cells expressing *KCS18* or *KCS20* accumulating relatively large amounts of C22:0 or C24:0, respectively (Figure 5C), suggesting that cotton KCS proteins have discrete substrate specificities.

In conclusion, we produced a binary mode of GA action as depicted in Figure 7E. GAs may act upstream of the VLCFA-ethylene pathway via activation of a large set of *GhKCS* genes (either *At* or *Dt* origin) (Figure 6B). GAs may function concomitantly in downstream steps that resulted in cellulose biosynthesis by promoting *CesA* expressions (Figure 6D). Clarification of this binary mode of GA action, in combination with progress in cotton genomics, may help elucidate molecular mechanisms controlling cotton growth and development.

MATERIALS AND METHODS

Materials and growth condition
Gossypium hirsutum cv. Xuzhou 142, *Gossypium arboreum* cv. Shixiya1 (SXY1) and *Gossypium raimondii* acc. D5-3 (CMD10)

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Figure 4. Preparations of *Saccharomyces* cerevisiae haploid *elo2Δelo3Δ* deletion strain over-expressing *ELO3*
(**A**) PCR experiments to verify the presence of *elo2::kanMX4* or *elo3::natMX4* cassettes using genomic DNA isolated from *elo2Δ*, *elo3Δ* or the *elo2Δelo3Δ* double mutant strains. Primers specific to *ELO2* promoter and *kanMX4* were designed as SP2 and AP2 (See Table S4 for primer sequences); Primers specific to *ELO3* promoter and *natMX4* were designed and SP3 and AP3. (**B**) Validating the prepared mutant strains on solid plates containing various chemicals or antibiotics, including Gal (galactose), Glu (glucose), G418, Clonat or FOA.
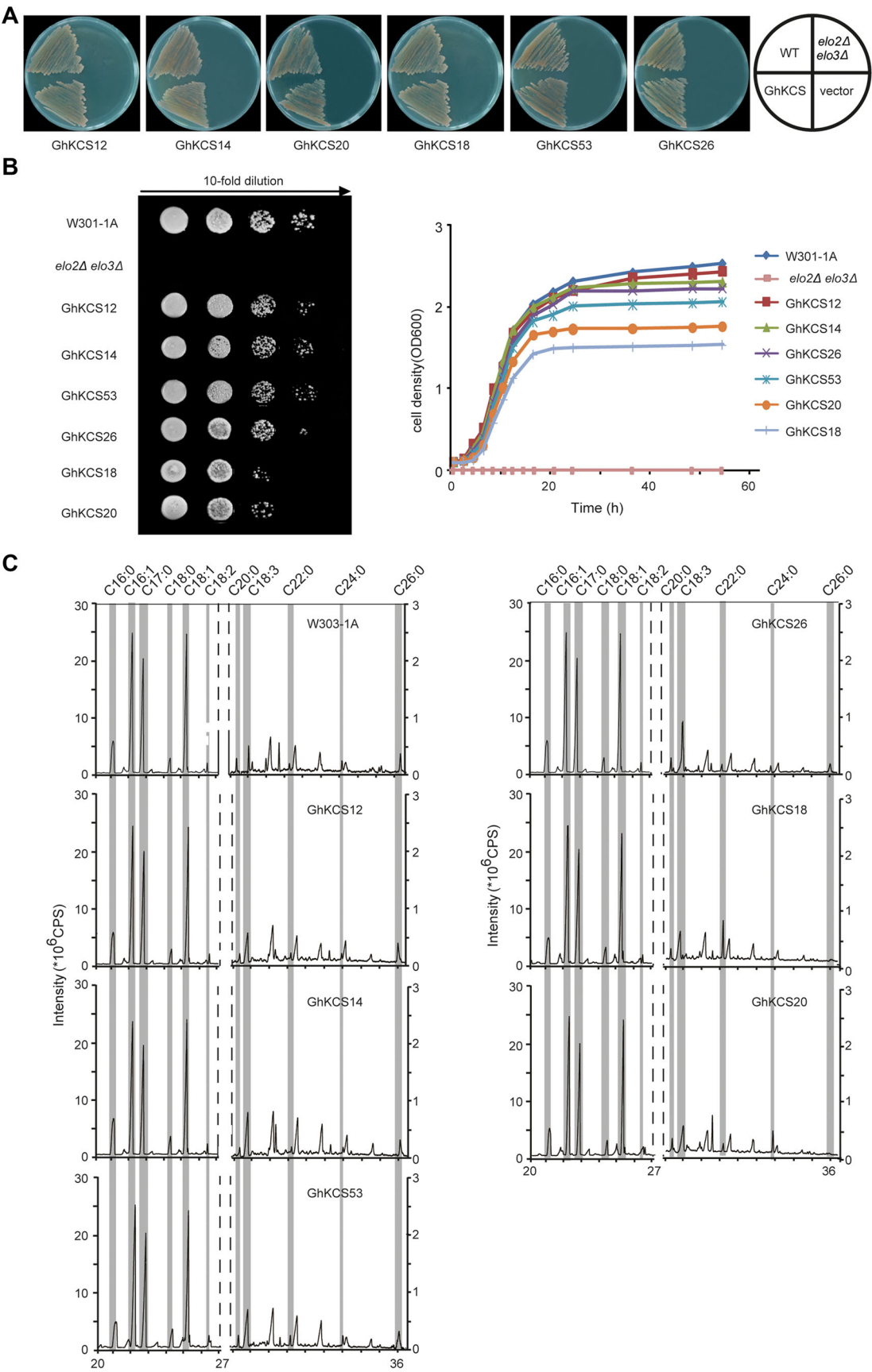
Figure 4. Continued.

Figure 5. Continued.

were grown in a climate-controlled greenhouse with a 16 h light and 8 h dark cycle at 25 °C (Liu et al. 2015). For *in vitro* ovule culture, 1 day post-anthesis (DPA) fresh ovules were excised from bolls on cotton plant and cultured directly on the surface of the liquid media (Shi et al. 2006). For RNA extraction, ovules collected from different growth stages and various tissues were frozen and stored in liquid nitrogen immediately after harvest.

Phylogenetic and evolution analysis

The sequence data used in this study were either sequenced as described above or collected from the National Center for Biotechnology Information (NCBI). A phylogenetic tree of deduced amino acid sequences was constructed using the neighbor-joining algorithm with default parameters in MEGA 5.0 (www.megasoftware.net). Multiple sequence alignments of the nucleotide were performed using ClustalX (Thompson et al. 1997).

RNA extraction and qRT-PCR analysis

Cotton ovules harvested at the indicated time were first frozen in liquid nitrogen before being ground to fine powder with a mortar and pestle using a modified hot borate method (Ji et al. 2003; Jin et al. 2013). Total RNA was extracted from ovules of three cotton species and cDNA was reverse-transcribed from 5 μg of total RNA as reported (Shi et al. 2006; Jin et al. 2013). For chemical treatment experiments, RNA samples were prepared from three independent ovule cultures in the presence of 1 μM GA$_3$, 5 μM C26:0 or without chemical supplementation (CK) for the indicated time. Primers for qRT-PCR analysis were listed in Table S4. Cotton UBQ7 was used as the internal control for all related figures.

In vitro ovule culture

Cotton bolls at 1 DPA were collected and sterilized with 10% sodium hypochlorite for 15 min. Ovules were excised from bolls and placed in liquid medium as described previously (Shi et al. 2006). 1 μM GA$_3$ or 5 μM C26:0 was added to the medium and ovules were cultured at 30 °C in darkness for the indicated time without agitation. 2.2 ppm ethylene was added in the head space of the flask used for ovule culture experiments as described previously (Shi et al. 2006). The cultured ovules were immediately collected for RNA extraction.

Construction of the yeast *elo2Δelo3Δ* double deletion mutant and functional complementation by cotton *KCSs*

In order to obtain haploid *Saccharomyces cerevisiae elo2Δelo3Δ* deletion strain (*Mat* a; *ade2Δ, can1-100, his3-11, leu2-3,*

trp1-1, ura3-1, elo3::natMX4, elo2::kanMX4), *elo3::natMX4* was amplified using genomic DNA prepared from BY4741 *elo3Δ* strain (*Mat a; his3Δ, leu2Δ, met15Δ, ura3Δ, elo3::kanMX4*) and subsequently transformed into *S. cerevisiae* W303-1A *elo2Δ* strain (*Mat* a; *ade2Δ, can1-100, his3-11, leu2-3, trp1-1, ura3-1, elo2::kanMX4*) carrying the pYES2-*ScELO3* vector. The *elo2Δelo3Δ* double mutant cells containing pYES2-*ScELO3* were selected on the plate supplemented with 200 μg/mL G418, 100 μg/mL Clonat and 2% (w/v) galactose, and subsequently transformed with individual *KCS* gene ligated into a *TRP1*-marked pYADE4 under the control of the *ADH* promoter. The transformants were selected on synthetic complete medium lacking tryptophan (SC-Trp) plates but containing 0.1% FOA. Cells were plated by 10-fold dilutions and were grown on the medium for 2 days at 30 °C before photographs were taken.

Fatty acid extractions and GC-MS analysis

One gram of fresh weight of wild type cells or *elo2Δelo3Δ* double deletion mutant cells complemented by individual cotton *KCSs* were ground to a fine powder with a mortar and pestle. Fatty acid were extracted by sulfuric acid: methanol (1:40, v/v) and derivatized by heating to 85 °C for 2 h (Browse et al. 1986). After cooling to room temperature, fatty acid methyl esters were extracted three times with hexane, dried under nitrogen gas and then concentrated to 0.5 mL. C17:0 fatty acid (heptadecanoic acid; Sigma-Aldrich), which does not exist in plants, was added before extraction to monitor sample recovery and also for quantitative analysis. 1 μL of each sample was analyzed on the Agilent 6890N GC system with the DB-225MS column (J&W) that operated with hydrogen carrier gas and a splitless inlet (injection temperature, 280 °C), according to the manufacturer's instructions. All fatty acids and hydroxylated fatty acids were identified by the Agilent 6890N GC system coupled to an HP 5973 mass selective detector using both the National Institute of Standards, and Technology and Wiley databases.

Analysis of Cellulose Content

Five milligram fiber cells separated from cultured ovules were ground to a fine powder with a mortar and pestle. Samples were treated with acetic nitric reagent for 30 min at 100 °C to obtain the insoluble residues. The pellets were washed with distilled water twice and hydrolyzed with 67% sulfuric acid. The cellulose content was quantified with anthrone reagent and suitable spectrophotometer at a wave-length of 620 nm against a reagent blank.

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Figure 5. Six *GhKCS* genes rescued the lethal growth phenotype of yeast *elo2Δelo3Δ* double mutant
(**A**) *elo2Δelo3Δ* double mutant yeast cells regain their ability to grow on solid FOA medium only when transformed with the identified *GhKCS* genes (labeled below each of the plates). WT, wild-type yeast strain W301-1A used for genetic transformations. (**B**) Detailed growth performance analysis of *elo2Δelo3Δ* double mutant yeast cell lines expressing various cotton *KCS* genes with comparisons to WT. Left, A series of 10-fold dilution that showed the density of the colonies expressing different cotton *KCS* genes on solid FOA media; Right, Growth curves of the same yeast cell lines grown in liquid FOA media. (**C**) GC-MS analyses of total fatty acids extracted from WT yeast cells or the *elo2Δelo3Δ* double mutant carrying various cotton *KCS* genes. The efficiency of the extraction process was monitored by external addition of fatty acid C17:0 and we adjusted the final sampling quantity for each cell line accordingly. The experiment was repeated three times with independent samples and only one representative graph was shown for each treatment.
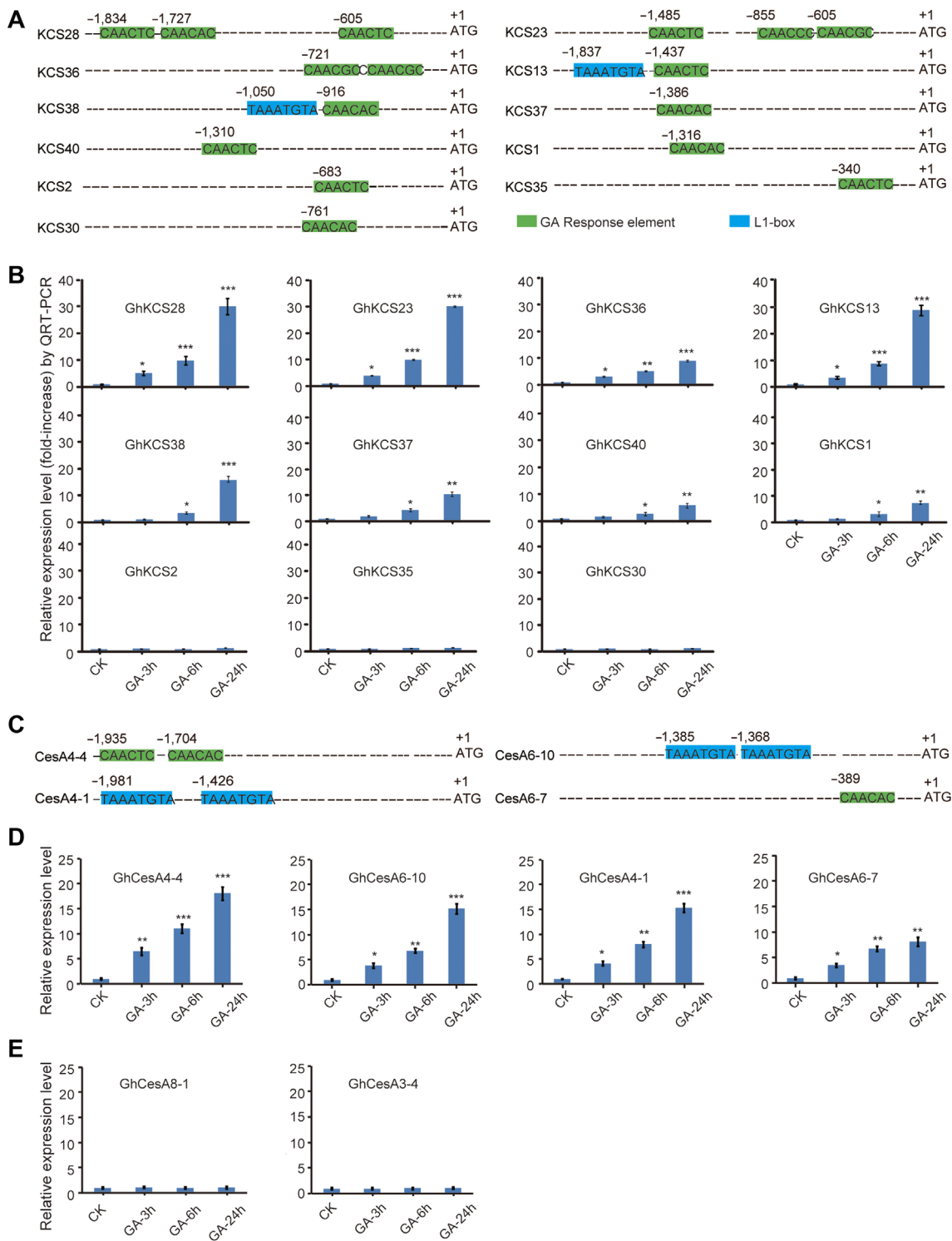
Figure 6. Exogenous gibberellin A₃ (GA₃) promotes the transcription of several *GhKCSs* and *GhCesAs* with GAREs on their promoters

(**A**) Analysis of *GhKCSs* with GAREs and L1 boxes present in their promoter regions. (**B**) GA activated significant transcription of most *GhKCS* genes with GAREs and L1 boxes in their promoter regions. (**C**) Analysis of *GhCesAs* with GAREs and L1 boxes present in their promoter regions. GAREs (marked by green color) and L1-box (blue color) were predicted using the PLACE and MEME softwares. (**D**) GA₃ promoted significant transcription activation of all four *GhCesA* genes with GAREs and L1 boxes. (**E**) The expression of the other two major *CesA* genes without L1 box or GARE were not induced by GA₃. Relative expression levels were determined after normalizing all data to that of CK ovules, which was set to 1.0. Statistical significance was determined using one-way analysis of variance software combined with Tukey's test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Electron Microscopic analyses of fiber cell wall thickness

Electron microscopic analyses were conducted essentially as previously described (Pugh et al. 2010). Wild-type cotton ovules harvested at 1 DPA were cultured for 7 d with either 1 μM GA$_3$, 5 μM C26:0 or 2.2 ppm ethylene added to the media. Cotton fibers from various treatments were fixed for 12 h in 2.5% glutaraldehyde. After three washes with distilled and deionized water, the fibers were incubated in 1.5% osmium tetraoxide for 4 h at room temperature. Samples were dehydrated in a series of ethanol (50%–100%) at 30 min intervals and epoxy ethane and then embedded in Epon resin for 48 h at 60 °C. Fibers at the base of the ovule were cut into 50 nm thin sections, stained with uranyl acetate and lead citrate, then photographed under a transmission electron microscope (Tecnai G2 20 TWIN, FEI).

Dry weight measurements

Wild-type cotton ovules were cultured as specified in the previous subsection for 7 d before harvest. The ovules were first rinsed in distilled and deionized water, heated to 100 °C for 5 min, transferred to 70% ethanol after cooling to room temperature and dried. The number of fibers (N) from each ovule treated with different chemicals was counted as described (Zhang et al. 2011) and fibers were removed from ovule surface for weight measurements (W1). According to the fiber length (L), dry weight per unit cell length was determined by the formula: $W = W_1/(N \times L)$.

AUTHOR CONTRIBUTIONS

G.X., G.H. and Y.Z. designed the phylogenetic analysis. G.X., K.W. and G.H. prepared RNA samples and performed PCR analysis. G.X. and G.H. performed complementary experiments in yeast. G.X., K.W. and G.H. performed the observation of cell wall. G.X. and K.W. wrote the manuscript. Y.Z. conceived and directed the project.

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Figure 7. Depicting the mode of gibberellin A (GA) action in an elongating cotton fiber cell
(A) Exogenous applications of GA$_3$ (1 μM), C26:0 (5 μM) and ethylene (2.2 ppm) promoted significant cotton fiber elongation. Cotton ovules were harvested at 1 DPA and cultured for 7 d before being fixed and measured under a light microscope. (B) Comparisons of dry fiber weight from ovule cultures after the same treatment as in (A). The experiment was repeated three times with five ovules each time and reported as mean ± SD for both (A) and (B). (C) Comparisons of cell wall thickness among fibers after the same treatment as in (A). CWT, cell wall thickness in μm; DIA, diameter of fiber cells in μm. Thirty fibers from three ovules were measured for each sample and each fiber was measured three times from different positions. The experiment was repeated three times and reported as mean ± SD. Scale bars = 2 μm. (D) Measurement of fiber cellulose content from ovule cultures after the same treatment as in (A). The experiment was repeated three times and reported as mean ± SD. (E) Proposed binary model of GA action during cotton fiber elongation. Statistical significance was determined using one-way analysis of variance software combined with Tukey's test. *P < 0.05; **P < 0.01; ***P < 0.001.
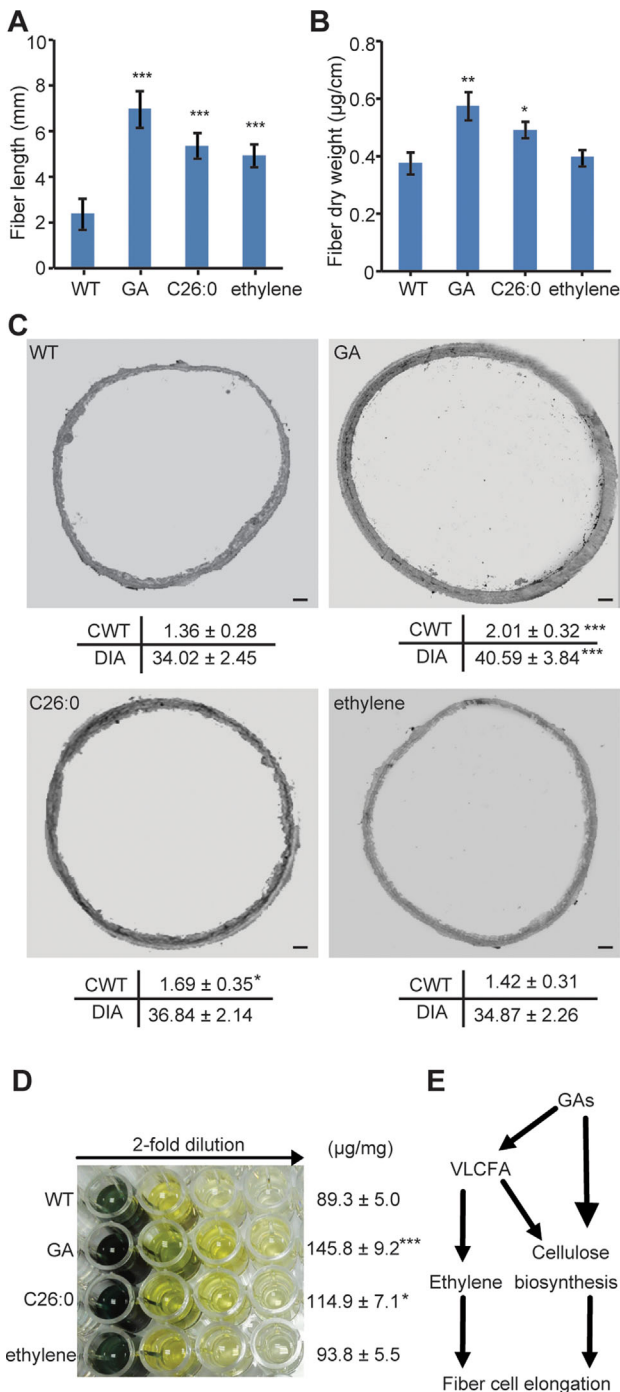
Figure 7. Continued.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Expression profiles of the other 25 *KCS* genes between the allotetraploid and the corresponding diploid genomes

(**A**) Transcripts of 15 *KCS* genes from the At or Dt subgenomes showed no genome- or subgenome-specificity when compared to their corresponding diploid orthologs in the tissues studied. (**B**) Ten *KCS* genes always expressed at almost undetectable levels in different cotton genomes or subgenomes in the tissues studied. R, root; S, stem; L, leaf; F, flower.

Figure S2. Exogenous BR and NAA promoted the expression of several *GhKCS* Genes

(**A**) Analysis of five *GhKCSs* with BES1 elements present in their promoter regions. (**B**) BR promoted significant transcription activation to four of these *GhKCSs*. (**C**) Analysis of two *GhKCSs* with AuxRE element present in their promoter regions. (**D**) NAA promoted significant transcription activation to these *GhKCSs*. Sequences highlighted in blue boxes are BES1 element and red boxes are AuxRE element predicted by the PLACE and MEME software. Relative expression levels were determined after normalizing all data to that of CK ovules, which was set to 1.0.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table S1. Analysis of *Gossypium hirsutum KCS* gene family and its orthologs in AA and DD cotton genomes

Table S2. Quantitative analysis of fatty acid composition from wild-type and *elo2Δelo3Δ* double mutant yeast cells genetically complemented with different cotton *KCSs* as reported in Figure 5

Table S3. Relative expression of *CesA* genes in ovules after application of C26:0 (**A**) and ethylene (**B**)

Table S4. A list of primers used in qRT-PCR experiments